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(54) **RESONANCE ENERGY TRANSFER ASSAY WITH SYNAPTOBREVIN SUBSTRATE MOIETY**

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C12Q 1/37 (2006.01)

(52) **U.S. Cl.**
CPC ***C12Q 1/37*** (2013.01); *C07K 2319/50* (2013.01); *C07K 2319/60* (2013.01); *G01N 2333/96486* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

Compositions and methods for analyzing intracellular BoNT protease activity, and especially BoNT/B, BoNT/G, BoNT/D, and/or BoNT/F protease activity are provided. Most preferably, cells express one or more recombinant hybrid proteins that include one or more fluorescent proteins and at least one BoNT protease recognition and cleavage sequence, and analysis is performed using FRET analysis.

14 Claims, 4 Drawing Sheets

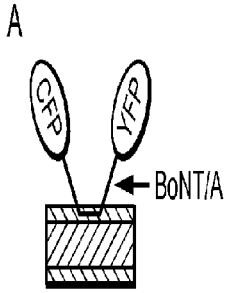
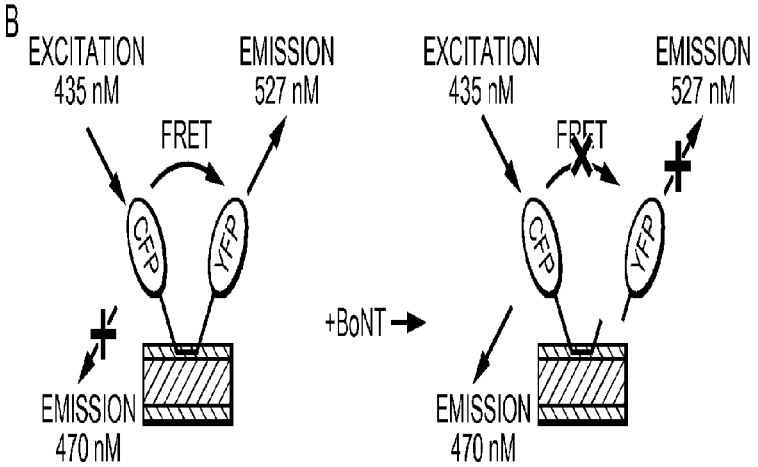


FIG. 1A
PRIOR ART



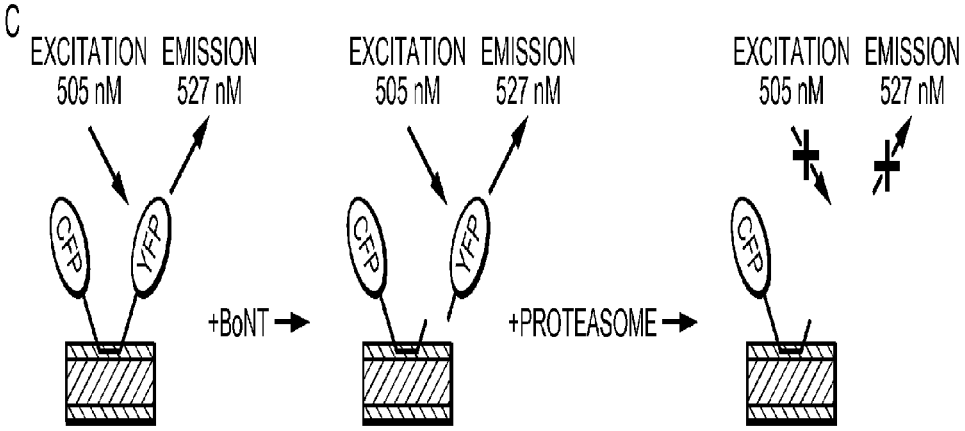
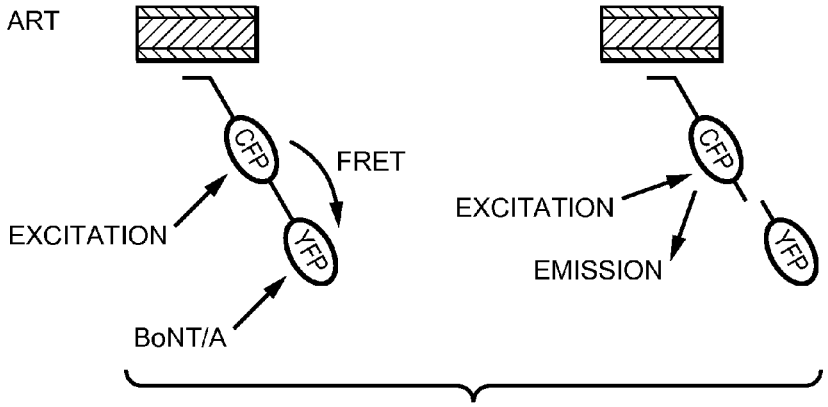


FIG. 1B
PRIOR ART



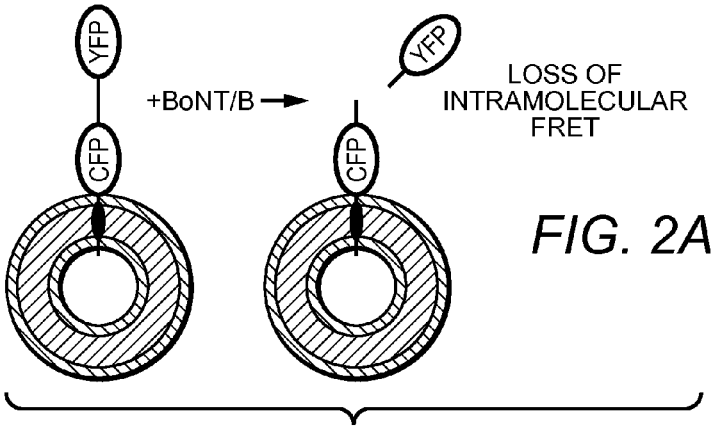


FIG. 2A

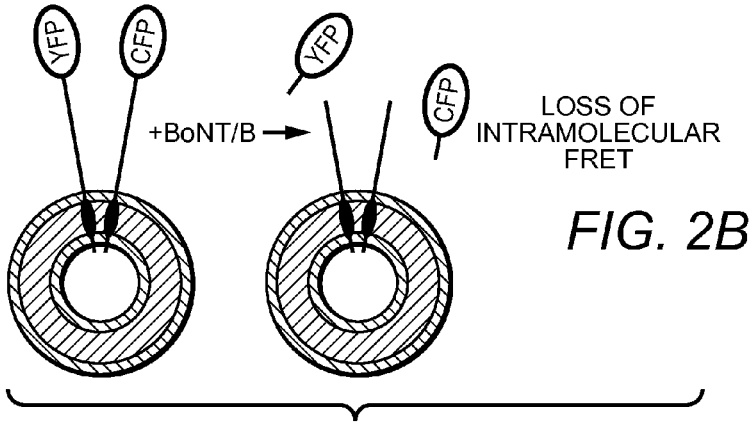
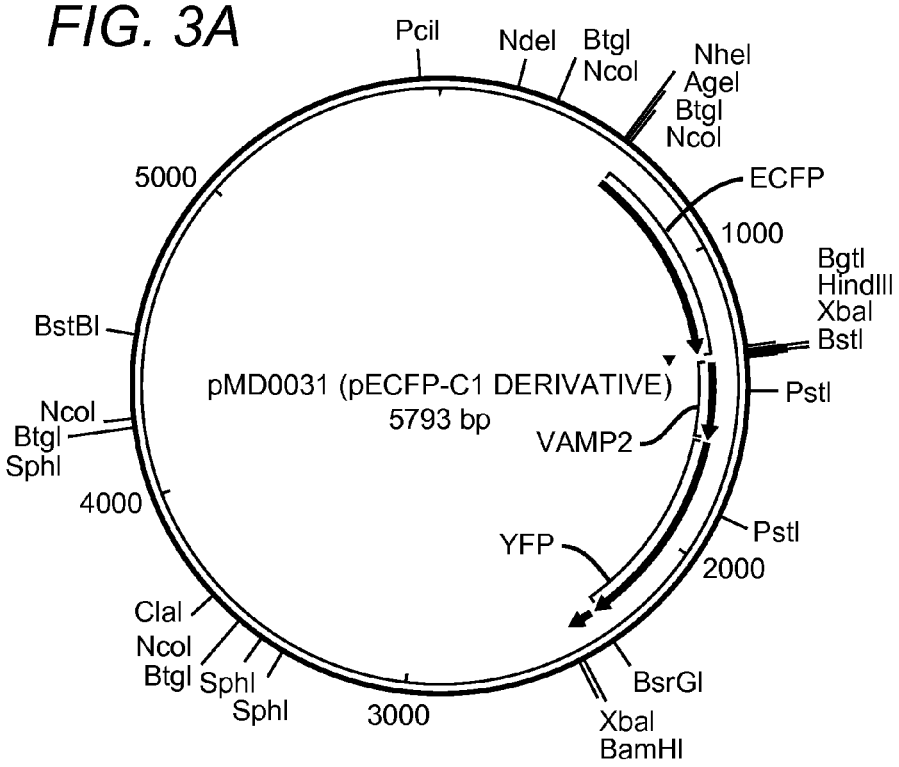


FIG. 2B

FIG. 3A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NdeI
BtgI
NcoI
NheI
AgeI
BtgI
NcoI
ECFP
1000
BgtI
HindIII
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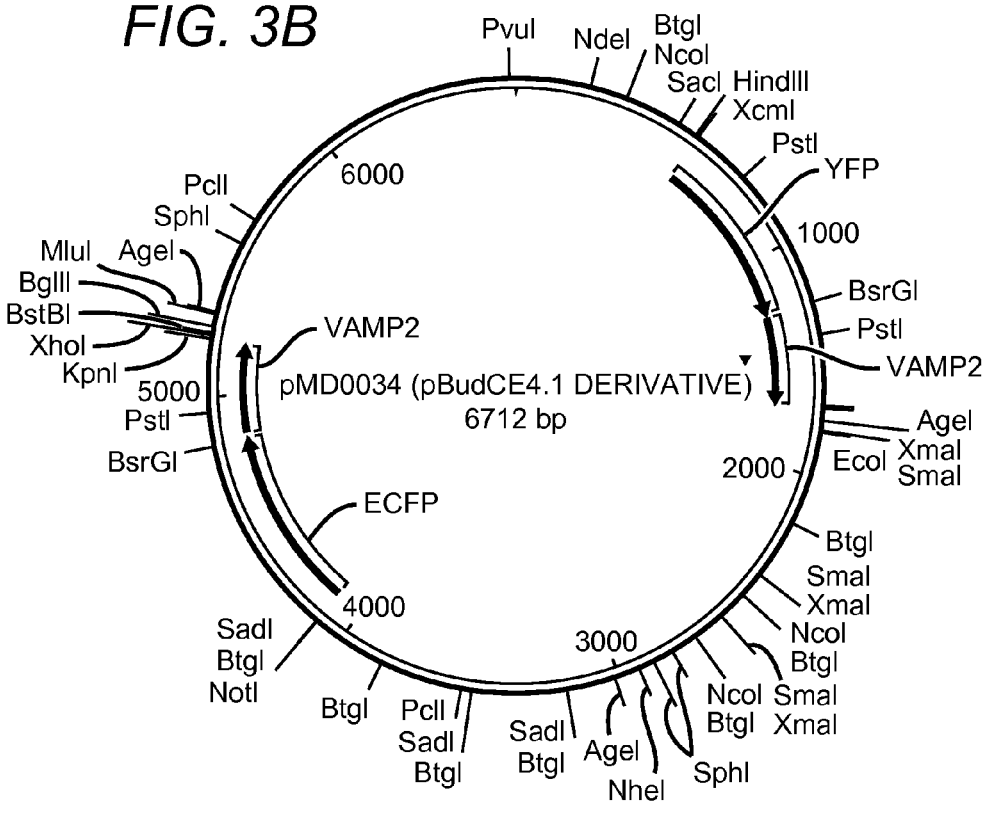
BstI
PstI
VAMP2
PstI
2000
YFP
BsrGI
XbaI
BamHI
3000
SphI
SphI
BtgI
NcoI
ClaI
4000
BtgI
NcoI
BstBI
5000
pMD0031 (pECFP-C1 DERIVATIVE)
5793 bp

FIG. 3B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NdeI
BtgI
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SacI
HindIII
XcmI
PstI
YFP
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BsrGI
PstI
VAMP2
AgeI
XmaI
SmaI
EcoI
2000
BtgI
SmaI
XmaI
NcoI
BtgI
NcoI
SmaI
XmaI
BtgI
3000
SphI
NheI
AgeI
SadI
BtgI
PciI
SadI
BtgI
BtgI
4000
NotI
SadI
BtgI
BsrGI
PstI
5000
KpnI
XhoI
BstBI
BglII
MluI
AgeI
SphI
PciI
6000
VAMP2
ECFP
pMD0034 (pBudCE4.1 DERIVATIVE)
6712 bp

FIG. 4A

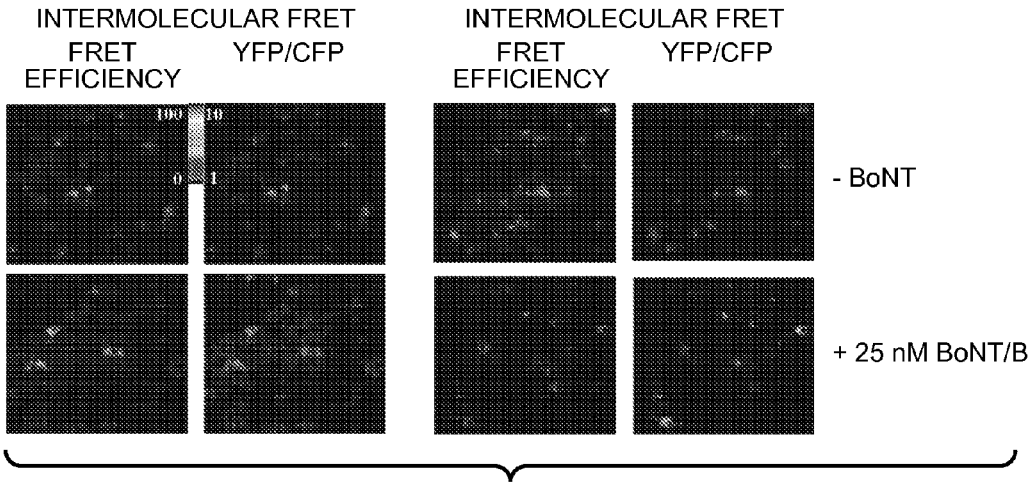
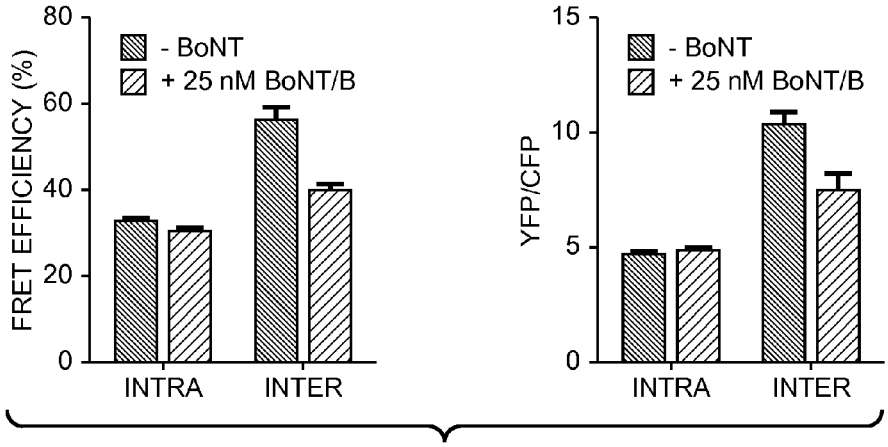

INTERMOLECULAR FRET
FRET EFFICIENCY
YFP/CFP
INTERMOLECULAR FRET
FRET EFFICIENCY
YFP/CFP
- BoNT
+ 25 nM BoNT/B

FIG. 4B


FRET EFFICIENCY (%)
80
60
40
20
0
- BoNT
+ 25 nM BoNT/B
INTRA
INTER
YFP/CFP
15
10
5
0
- BoNT
+ 25 nM BoNT/B
INTRA
INTER

RESONANCE ENERGY TRANSFER ASSAY WITH SYNAPTOBREVIN SUBSTRATE MOIETY

This application claims priority to U.S. provisional application with the Ser. No. 61/252,315, which was filed Oct. 16, 2009.

FIELD OF THE INVENTION

The field of the invention is Förster resonance energy transfer (FRET) assays for protease activity, especially protease assays for *Botulinum* neurotoxins BoNTs that cleave synaptobrevin.

BACKGROUND OF THE INVENTION

Botulinum neurotoxins (BoNTs) are extremely toxic proteins and can be classified into distinct subgroups based, inter alia, on peptide sequence and/or substrate specificity. All of the naturally occurring BoNTs (BoNT/A-G) are composed of a heavy chain that mediates toxin entry into a target cell and a light chain with zinc-dependent protease activity that hydrolyzes selected SNARE proteins that mediate fusion of neurotransmitter vesicles to the membrane that forms part of the synaptic cleft.

For example, the light chain of BoNT/A hydrolyzes with high specificity SNAP-25, which is required for vesicle-mediated exocytosis of acetylcholine into the synaptic cleft. Known assays for such hydrolytic activity include those described in our copending International application (WO 2009/035476), which is incorporated by reference herein. Here, a fluorophore and a quencher are covalently linked to the respective ends of a peptide sequence that includes, for example, the SNAP-25 sequence. Cleavage by BoNT/A (or other BoNTs with a substrate specificity towards SNAP-25) will result in physical separation of the cleavage products and so reduce fluorescence quenching, which can then be quantified. Among other choices, it is often preferred that such assay is performed as an in vitro solid-phase based assay.

While such assay is conceptually simple and can be used to readily determine BoNT/A, BoNT/C, or BoNT/E activity, such assay can not be simply modified to a cell-based assay for determination of BoNT/B, BoNT/D, BoNT/F, or BoNT/G activities by replacing the SNAP-25 motif with a SNARE domain as the SNARE domain includes a membrane spanning sub-domain that would place the N-terminal fluorophore into a vesicle lumen. In such case, only diffusion of the fluorescence signal would be observed as has been reported elsewhere (Dong et al. PNAS (2004), Vol. 101, No. 41, 14701-14706; or U.S. Pat. App. No. 2006/0134722).

Therefore, there is still a need for improved BoNT assays, and especially cell-based assays for BoNTs that cleave synaptobrevin.

SUMMARY OF THE INVENTION

The present invention is directed to various compositions and methods of analyzing BoNT protease activity, and especially BoNT/B, BoNT/G, BoNT/D, and/or BoNT/F protease activity in a cell-based system using fluorescence resonance energy transfer. Most preferably, the cells express one or more recombinant hybrid proteins together with at least one BoNT protease recognition and cleavage sequence, wherein the hybrid protein further comprises a transmembrane domain that is not cleavable by the BoNT protease and that directs the hybrid protein to an intracellular synaptic vesicle.

In one aspect of the inventive subject matter, a cell-based method of measuring protease activity of a BoNT protease, in which in one step a transfected cell is provided that produces (I) a hybrid protein having a structure of A-B-C-D or (II) two hybrid proteins having a structure of A-C-B and A-C-D, respectively, wherein A is a transmembrane domain that is not cleavable by the BoNT protease, B is a first fluorescent protein, C is a BoNT protease recognition and cleavage sequence, and D is a second fluorescent protein. IN another step, the transfected cell is contacted with a BoNT protease under conditions to allow the cell to take up the BoNT protease, and in yet another step, fluorescence is measured of at least one of the first and second fluorescent proteins in the transfected cell.

Most preferably, the transfected cell is a neuronal cell, a neuroendocrine tumor cell, a hybrid cell, or a stem cell. It is further generally preferred that A includes a transmembrane domain from synaptobrevin, synaptophysin, synapsin I, synapsin II, and/or synapsin III, and/or that C includes at least two of a BoNT/B, a BoNT/G, a BoNT/D, and a BoNT/F protease recognition and cleavage sequence. While not limiting to the inventive subject matter, it is further preferred that a peptide linker is disposed between one or more of A and C, A and B, C and B, and C and D, and that the linker has a length of equal or less than 12 amino acids. Additionally, it is contemplated that the transfected cell may be contacted with a putative or known BoNT inhibitor prior to contacting the transfected cell with the BoNT protease. In especially preferred aspects, the transfected cell produces two hybrid proteins.

In exemplary embodiments, the hybrid protein having the structure of A-B-C-D has a sequence according to SEQ ID NO:2, the hybrid protein having the structure of A-C-B has a sequence according to SEQ ID NO:4, and the hybrid protein having the structure of A-C-B has a sequence according to SEQ ID NO:6.

Therefore, and viewed from a different perspective, a recombinant nucleic acid includes a sequence that encodes (I) a hybrid protein having a structure of A-B-C-D or (II) two hybrid proteins having a structure of A-C-B and A-C-D, respectively, wherein A is a transmembrane domain that is not cleavable by the BoNT protease, B is a first fluorescent protein, C is a BoNT protease recognition and cleavage sequence, and D is a second fluorescent protein. Most preferably, A comprises a transmembrane domain from synaptobrevin, synaptophysin, synapsin I, synapsin II, and/or synapsin III, and/or C comprises at least two of a BoNT/B, a BoNT/G, a BoNT/D, and a BoNT/F protease recognition and cleavage sequence. Where desired, at least one additional sequence may be provided that encodes a peptide linker that is disposed between at least one of A and C, A and B, C and B, and C and D, wherein the linker has a length of equal or less than 12 amino acids.

In especially preferred aspects, the recombinant nucleic acid encodes the two hybrid proteins. In exemplary nucleic acids, the hybrid protein having the structure of A-B-C-D is encoded by a sequence according to SEQ ID NO:1, the hybrid protein having the structure of A-C-B is encoded by a sequence according to SEQ ID NO:3, and the hybrid protein having the structure of A-C-B is encoded by a sequence according to SEQ ID NO:5.

Consequently, the inventors also contemplate a cell transfected with the nucleic acid presented herein, and it is generally preferred that the cell is stably transfected with the

nucleic acid. Especially suitable cells include neuronal cells, neuroendocrine tumor cells, hybrid cells, and stem cells. Furthermore, it is typically preferred that the cell comprises a nucleic acid that encodes the two hybrid proteins having the structure of A-C-B and A-C-D.

Various objects, features, aspects and advantages of the present invention will become more apparent from the following detailed description of preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWING

Prior Art FIGS. **1**A-**1**B are known FRET assays for BoNT protease activity in which two fluorescent proteins are separated by a SNAP25 recognition and cleavage sequence.

FIGS. **2**B-**2**B are schematic illustrations for intramolecular (**2**A) and intermolecular (**2**B) FRET assays for BoNT protease activity according to the inventive subject matter.

FIGS. **3**A-**3**B are exemplary vector maps for recombinant intramolecular (**3**A) and intermolecular (**3**B) FRET constructs according to the inventive subject matter.

FIG. **4**A depicts exemplary FRET results for intramolecular (left panel) and intermolecular (right panel) FRET analysis according to the inventive subject matter.

FIG. **4**B is a graphic representation of the results from the experiments of FIG. **4**A.

DETAILED DESCRIPTION

According to the present invention a cell-based FRET assay for BoNT (and especially for BoNT/B, BoNT/D, BoNT/F, or BoNT/G) is provided in which a cell is transfected cell such that the cell produces (a) a single hybrid protein having a structure of A-B-C-D, or (b) two distinct hybrid proteins having a structure of A-C-B and A-C-D, respectively, in which A is a transmembrane domain, B is a first fluorescent protein, C is BoNT protease recognition and cleavage sequence, and D is a second fluorescent protein, where most typically, B and D allow for a FRET assay.

It should be appreciated that the hybrid protein(s) that are formed in the so transfected cells include a transmembrane domain. Therefore, these proteins are expected to locate to intracellular vesicles and to so present a vesicle-bound substrate. Upon exposure of the cells with BoNT, heavy chain-mediated endocytosis of the BoNT into the transfected cell is followed by presentation of the light chain on the outer surface of the vesicle, allowing the protease activity of the light chain to cleave the cleavage sequence of the hybrid protein(s), thus reducing FRET and providing a quantifiable signal. Therefore, it should be appreciated that the compositions and methods presented herein allow for a cell-based assay in which uptake, processing, and proteolytic activity can be monitored under conditions that closely resemble the natural conditions.

In contrast, as schematically depicted in Prior Art FIG. **1**A, a BoNT/A test system with a hybrid protein is shown in A. The hybrid protein has first and second fluorescence proteins (CFP and YFP, respectively) covalently coupled to the respective termini of an intermediate peptide sequence that also includes a SNAP-25 sequence (which is the substrate for the BoNT/A light chain protease). Excitation of the CFP results in FRET-mediated fluorescence emission of YFP, thus creating a specific spectral fluorescence signature as schematically illustrated in B. Upon incubation with BoNT/A, the SNAP-25 sequence is hydrolyzed and YFP is released from the hybrid molecule (the remainder of which is still bound to a membrane or other solid phase) as depicted in C. Alternatively, or additionally, excitation and emission may be followed only using YFP, which when separated from the hybrid protein, will ultimately be processed in the proteasome complex. Similarly, as shown in Prior Art FIG. **1**B, a hybrid protein has first and second fluorescence proteins (CFP and YFP, respectively) covalently coupled to the respective termini of an intermediate peptide sequence that also includes a SNAP-25 sequence. The hybrid protein is associated to the outside of the vesicle via the cysteine rich domain of the SNAP-25 sequence. Once more, upon cleavage of the SNAP-25 linker between the CFP and YFP, FRET is no longer available and fluorescence can be measured either as loss in FRET or ultimately loss in YFP as described above.

While such systems provide various advantages, it should be readily apparent that that where the SNAP-25 sequence is replaced by a synaptobrevin (VAMP), the presence of the transmembrane sub-domain in synaptobrevin will lead to physical separation of the CFP and YFP by the vesicle (or other) membrane, thus abolishing any FRET between the CFP and YFP as is shown in FIG. 9B of U.S. Pat. App. No. 2006/0134722.

To overcome these difficulties, the inventors now have prepared hybrid molecules suitable for intramolecular FRET in which one fluorescent protein (or other reporter) is positioned between the transmembrane sub-domain and the BoNT protease recognition and cleavage sequence, and wherein another fluorescent protein (or other reporter) is positioned following the BoNT protease recognition and cleavage sequence. Additionally, the inventors have also prepared pairs of hybrid molecules suitable for intermolecular FRET in which both hybrid molecules have a respective fluorescent protein coupled to respective sequences that include a transmembrane domain and a BoNT protease recognition and cleavage sequence.

As used herein, the term "transmembrane domain" refers to any molecular moiety that is capable of insertion into a plasma membrane in a manner such that at least a portion of the moiety extends into (and more typically across) the lipid bilayer. Thus, a moiety that only externally contacts (e.g., via ionic or electrostatic interaction) the outer surface of the plasma membrane is not considered a transmembrane domain under the definition provided herein. Thus, especially preferred transmembrane domains include hydrophobic polypeptide domains that extend into (and more typically across) the plasma membrane. Most typically, preferred transmembrane domains comprise a (typically recombinant) polypeptide. However, it should be recognized that various alternative elements (e.g., N-terminal palmitoylation) will also fall within the scope of the definition provided herein.

As also used herein, the term "BoNT recognition and cleavage sequence" refers to any molecular moiety that can be bound and cleaved by a BoNT protease. It is generally preferred that the BoNT recognition and cleavage sequence comprises a synaptobrevin polypeptide or portion thereof, which is typically a recombinant polypeptide.

In one especially preferred aspect of the inventive subject matter, contemplated recombinant nucleic acids may include a sequence that encodes (I) a hybrid protein having a structure of A-B-C-D or (II) at least one of two hybrid proteins having a structure of A-C-B and having a structure of A-C-D, respectively, where A is a transmembrane domain, B is a first fluorescent protein, C is a BoNT recognition and cleavage sequence, and D is a second fluorescent protein. Most preferably, where the sequence encodes two hybrid proteins, expression of the two hybrid proteins is under the

control of respective promoters (typically, but not necessarily, having the same strength and same regulatory control mechanism).

Most typically, the transmembrane domain is selected such as to allow insertion of the recombinant protein(s) into the membrane of synaptic vesicles. Therefore, it is generally preferred that the transmembrane domain is the transmembrane domain of synaptobrevin, synaptophysin, synapsin I, synapsin II, and/or synapsin III, or any portion thereof that still confers anchoring of the recombinant protein into the membrane. However, in alternative aspects of the inventive subject matter, it is contemplated that various other transmembrane domains are also deemed suitable so long as such domains will anchor the recombinant protein to one or more intracellular membranes. There are numerous transmembrane domains known in the art, and all of those are deemed suitable for use herein. The person of ordinary skill in the art will readily be able to identify a domain as a transmembrane domain (e.g., via publication and description of the domain, or via computational domain analysis). Of course, suitable domains naturally occurring domains as well as mutated forms thereof (e.g., forms with one or more transitions, transversions, insertions, deletions, inversions, etc.). Moreover, additionally contemplated transmembrane domain may also be entirely synthetic and based on computational analysis.

Similarly, it should be appreciated that the transmembrane domain may also be replaced by another polypeptide moiety that allows at least temporary anchoring of the hybrid protein to a membrane such that the remainder of the hybrid protein is exposed to the cytosol. Anchoring may be mediated by various (typically non-covalent) interactions, including ionic, hydrophobic, and/or electrostatic interactions. Still further contemplated transmembrane domains also include non-protein transmembrane domains. For example, especially preferred alternative transmembrane domains will include those in which a hydrophobic group (e.g., sterol, hydrocarbon, etc.) is attached to the protein, and particularly a palmitoyl group. Such groups may be added intracellularly (e.g., via palmitoylation signal) or in vitro using various synthetic schemes.

It should further be appreciated that suitable transmembrane domains will preferably not include a BoNT protease cleavage site and/or a BoNT protease recognition site and thus only be acting as a transmembrane anchor for the recombinant protein. Therefore, suitable transmembrane domains may include full-length (or substantially full-length) synaptobrevin that has been sufficiently mutated to eliminate the cleavage site and/or recognition site. Alternatively, the synaptobrevin (or other transmembrane domain) may be truncated such that at least the cleavage site and/or recognition site is removed. Moreover, while the above is directed to single transmembrane domains, it should be appreciated that more than one transmembrane domains are also deemed appropriate (which are preferably coupled to each other via a spacer).

With respect to first and second fluorescent proteins it is generally contemplated that all known fluorescent proteins are suitable for use herein so long as such proteins can be used as specific and distinct signal generation moieties. However, it is particularly preferred that the signal generation moieties are fluorescent proteins that are suitable for FRET. For example, first and second fluorescent proteins can be Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) and their respective modifications, respectively. Of course, and as already noted above, the fluorescent proteins may be modified to include one or more specific characteristics (e.g., spectral) or be truncated to a specific size. Among other choices, contemplated fluorescent proteins include various blue fluorescent proteins (e.g., EBFP, EBFP2, Azurite, mKalama1), various cyan fluorescent proteins (e.g., ECFP, Cerulean, CyPet), various green fluorescent proteins (e.g., AcGFP1, ZsGreen1), and various yellow fluorescent protein derivatives (e.g., YFP, Citrine, Venus, YPet).

Similarly, it should be appreciated that the BoNT protease recognition and cleavage sequence may vary considerably, so long as such sequence is still recognized and hydrolyzed by a BoNT light chain. For example, the BoNT protease recognition and cleavage sequence may be of human, rat, or murine origin, may be present in oligo-multimeric form, and may be further specifically modified to facilitate or at least partially inhibit cleavage. Alternatively, the BoNT protease recognition and cleavage sequence may also be modified to allow identification of one or more specific BoNT subtypes (e.g., BoNT/B, D, F, and/or G, as well tetanus toxin) by preferential or exclusive cleavage. Of course, it should be recognized that all isoforms and mutants of BoNT protease recognition and cleavage sequences are also deemed suitable for use in conjunction with the teachings presented herein so long as such forms and mutants are also cleavable by one or more BoNT proteases. For example, suitable protease recognition and cleavage sequences include those from VAMP (Synaptobrevin) 1, 2, 3, 4, 5, 6, 7, or 8, and exemplary sequences are listed below where the recognition and cleavage domain is in regular type font, the transmembrane domain is in cursive type font, and where the actual cleavage positions for the respective BoNT proteases are underlined (QK: BoNT/F; KL: BoNT/D; QF: BoNT/B and TeTN; AA: BoNT/G):

Rat Vamp2 Protein sequence (SEQ ID NO: 7):

SEQ ID NO: 7

MSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD

KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY*WWKNLKMMIILGVICAIILIII*

VYFST

Mouse Vamp2 Protein sequence (SEQ ID NO: 8):

(SEQ ID NO: 8)

MSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD

KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY*WWKNLKMMIILGVICAIILIII*

VYFST

Human Vamp2 Protein sequence (SEQ ID NO: 9):

-continued

(SEQ ID NO: 9)

MSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD

KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY*WWKNLKMMIILGVICAIILIIII*

VYFST

Of course, it should be noted that the above sequences merely serve as examples for the sequences from which the transmembrane domain and the BoNT protease recognition and cleavage sequences can be obtained from. Thus, it is also noted that numerous alternative sequences other than synaptobrevin are also contemplated particularly if they can be cleaved by a naturally occurring or a synthetic or designer BoNT, including SNAP-25 and mutant forms thereof.

It should further be appreciated that one or more of the transmembrane domain, the first and second fluorescent proteins, and the BoNT protease recognition and cleavage domain may be truncated while retaining the respective function (i.e., transmembrane anchor, fluorescence, BoNT protease recognition and cleavage). Moreover, it should be appreciated that one or more amino acids in the above elements may be deleted or replaced by one or more other amino acids, typically in a conserved fashion.

Moreover, it is especially contemplated that the additional amino acids may be added as spacers between one or more of the transmembrane domain, the first and second fluorescent proteins, and the BoNT protease recognition and cleavage domain. Such spacers may be included to provide further steric flexibility, increase distance between the elements, etc. Typically, suitable spacers will have a length of between 1-100 amino acids, more typically between 2-50 amino acids, and most typically between 3-12 amino acids. Where the recombinant protein is used for FRET assays, shorter spacers are generally preferred. Still further, it is noted that additional domains may be provided to impart further desired functions. For example, suitable additional domains will include affinity tags for ease of isolation or antibody-based labeling, cell trafficking to direct the recombinant protein into a desired compartment, etc.

With respect to the transfected cells expressing the hybrid protein it is generally preferred that the cell is stably transfected. Nevertheless, transient transfection is also contemplated. There are numerous promoter structures known in the art, and all of those are generally deemed suitable for use herein. However, it is especially preferred that the expression is inducible from the promoter. In further contemplated aspects, expression may also be constitutively. FIG. **3**A depicts an exemplary vector map for an expression construct of a hybrid protein having a structure of A-B-C-D, and FIG. **3**B depicts an exemplary vector map for expression of two hybrid proteins having a structure of A-C-B and A-C-D, respectively.

Particularly preferred cells for transfection include neuronal cells (e.g., astrocytes, dendrocytes, glia cells, etc.) and stem cells (e.g., adult pluripotent, or adult germ line layer, or adult progenitor). However, numerous other non-neuronal cells, including human, rodent, insect cells, and even yeast and bacterial cells are also contemplated herein.

Consequently, the inventors contemplate a cell-based method of measuring protease activity of a BoNT protease in which in one step a transfected cell is provided that produces (I) a hybrid protein having a structure of A-B-C-D or (II) two hybrid proteins having a structure of A-C-B and A-C-D, respectively, wherein A is a transmembrane domain, B is a first fluorescent protein, C is a BoNT recognition and cleavage sequence, and D is a second fluorescent protein. In exemplary aspects of the inventive subject matter, the hybrid protein having a structure of A-B-C-D has a sequence according to SEQ ID NO:2, which is preferably encoded by a nucleic acid having sequence according to SEQ ID NO:1. Where the hybrid proteins have a structure of A-C-B and A-C-D, the protein sequences will preferably be as shown in SEQ ID NO:4 and SEQ ID NO:6, which are preferably encoded by a nucleic acid having sequence according to SEQ ID NO:3 and SEQ ID NO:5, respectively. Of course, and as already noted earlier, all mutant forms of the above sequences are also expressly contemplated herein, so long as such mutant forms retain the respective functions as noted above. In another step, the transfected cell is contacted with a BoNT protease under conditions to allow the cell to take up the BoNT protease, and in yet another step, fluorescence is measured from at least one of the first and second fluorescent proteins in the transfected cell.

Depending on the particular requirements and conditions, contemplated cell based assays may be performed as depicted in FIG. **2**A in which the hybrid protein is a single polypeptide chain having an N-terminal transmembrane domain, followed by a CFP, which is in turn followed by a BoNT protease recognition and cleavage sequence, which is in turn followed by a (preferably terminal) YFP. Expression of the hybrid protein and subsequent translocation of the hybrid protein to the membrane of an intracellular vesicle will result in the presentation of the hybrid protein on the outside of the vesicle. The protease activity of BoNT/B will then intracellularly cleave the cleavage sequence, thus releasing the YFP from the hybrid protein. Consequently, quenching is reduced and fluorescence of the YFP is detectable in diffused form from the cell.

Alternatively, as shown in FIG. **2**B, two separate hybrid proteins are formed in the cell, each having an N-terminal transmembrane domain, followed by a BoNT protease recognition and cleavage sequence, which is in turn followed by a (preferably terminal) YFP and CFP, respectively. Expression of the hybrid proteins and subsequent translocation of the hybrid proteins to the membrane of an intracellular vesicle will result in the presentation of the hybrid proteins on the outside of the vesicle. The protease activity of BoNT/B will then intracellularly cleave the cleavage sequences, thus releasing YFP and CFP from the hybrid protein. Consequently, quenching is reduced and fluorescence of the YFP and CFP is detectable in diffused form from the cell. Remarkably, the respective hybrid proteins co-locate on the vesicular membrane in such a manner as to allow for FRET. Exemplary results for such assays are depicted in the calculated fluorescence microphotographs of FIG. **4**A and the corresponding bar graph representations of FIG. **4**B. As can be readily taken from these figures, the FRET assay performed well in the intermolecular FRET assay and less satisfactorily in the intramolecular FRET assay. However, it is expected that routine experimentation will also provide satisfactory intramolecular FRET assay results.

EXAMPLES

Cloning of Intramolecular Construct

The intramolecular FRET construct, pMD0031 (FIG. **3**A), was constructed in pEGFP-C1 (Clontech, Mountain View, Calif.). Three DNA fragments—an N-terminal fragment of rat Vamp2 from the start to amino acid 92, full length YFP without a stop codon, and a C-terminal fragment of rat Vamp2 from amino acid 93 to the stop—were amplified by polymerase chain reaction (PCR). An EcoRI restriction site was engineered onto the 5' end of the N-terminal Vamp2 fragment and a SerGlyGly (TCGGGAGGC) linker and the first 12 nucleotides of the YFP were engineered onto the 3' end. The YFP fragment had the last 13 nucleotides of the N-terminal Vamp2 fragment and the same SerGlyGly linker as the N-terminal Vamp2 fragment engineered onto the 5' end and a second SerGlyGly (AGCGGCGGT) linker and the first 9 nucleotides of the C-terminal Vamp2 fragment engineered onto the 3' end. The C-terminal Vamp2 fragment had the last 12 nucleotides of YFP without a stop and the same SerGlyGly linker as the YFP fragment engineered onto the 5' end and a BamHI restriction site engineered onto the 3' end.

These three fragments were then combined using splice overlap extension (SOE) PCR to create a single fragment consisting of an EcoRI restriction site, the N-terminal fragment of rat Vamp2 (amino acids 1-92), a SerGlyGly linker, YFP without a stop, a second SerGlyGly linker, the C-terminal fragment of rat Vamp2 (amino acids 93-stop), and an BamHI restriction site. This fragment and pECFP-C1 were then digested with EcoRI and BamHI, ligated together, and transformed into DH5α *E. coli*. The final construct insert was then fully sequenced.

Cloning of Intermolecular Construct

The intermolecular FRET construct, pMD0034 (FIG. **3**B), was constructed in pBudCE4.1 (Invitrogen, Carlsbad, Calif.). The YFP rat Vamp2 fusion was generated by amplifying two fragments by PCR. The first fragment was YFP without a stop with an engineered HindIII restriction site on the 5' end and a SerGlyGly linker (AGTGGAGGC) and the first 9 nucleotides of rat Vamp2 engineered on the 3' end. The second fragment was full length rat Vamp2 with the last 9 nucleotides of YFP and the same SerGlyGly linker engineered onto the 5' end and an XbaI restriction site engineered onto the 3' end. These two fragments were then combined using SOE PCR to create a YFP, SerGlyGly linker, full length Vamp2 fragment. The fragment and pBudCE4.1 was then digested with HindIII and XbaI, ligated together, and transformed into DH5 α *E. coli*. The CFP rat Vamp2 fusion was created similarly but contained a CFP without a stop, a NotI restriction site on the 5' end, and a KpnI site on the 3' end. The final construct was then fully sequenced.

Cell Culture and FRET Assay

Analysis of FRET efficiency, YFP/CFP fluorescence ratios, and BoNT/B sensitivities of the BoNT/B reporters was performed in cells in vitro. More specifically, Neuro2A cells were grown in a 96-well plate to 70% confluency (~2000 cells/well) and transiently transfected using Lipofectamine 2000 (Invitrogen), with the intra- or intermolecular BoNT/B reporters. After 24 h, cells were incubated in the presence or absence of 25 nM BoNT/B at 37° C. for 72 h in 100 μl of phenol red-free MEM medium.

Semi-automated FRET or total YFP and CFP fluorescence measurements were performed using a Nikon TE2000-U fluorescent microscope with 200× magnification and Nikon NIS Elements 3.4 software. For FRET measurements, coefficients-A and -B (acceptor and donor) were calculated at 0.03 and 0.73 respectively, using a specific bleed-through method. FIG. **4**A depicts randomly selected fields pseudocolored for FRET efficiency or the YFP/CFP fluorescence ratio. YFP/CFP ratios were calculated from emissions collected upon direct excitement of each fluorophore. As can be seen from the graphic representation in FIG. **4**B, the intermolecular BoNT/B reporter approach was significantly more sensitive for detection of BoNT/B in living cells. 30 randomly selected cells per condition were analyzed for FRET efficiency (FIG. **4**A, left panels) or YFP/CFP fluorescence ratios (FIG. **4**A, right panels) in the presence or absence of 25 nM BoNT/B. Indeed, such results were entirely unexpected as effective intermolecular FRET not only required balanced expression of the two fluorescent proteins, but also co-location of the recombinant proteins in corresponding quantities. The average signal from the 30 cells from 5 microscopic fields on 3 different wells is shown. Cells exhibiting over-saturated fluorescence were excluded.

Thus, specific embodiments and applications of BoNT assays have been disclosed. It should be apparent, however, to those skilled in the art that many more modifications besides those already described are possible without departing from the inventive concepts herein. The inventive subject matter, therefore, is not to be restricted except in the spirit of the appended claims.

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aagctatcgg aactggatga tcgcgcagat gccctccagg caggggcctc ccagtttgaa 960

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<210> SEQ ID NO 6
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<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Recombinant protein encoded by pMD0034 ECFP
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<222> LOCATION: (1)..(239)
<223> OTHER INFORMATION: Enhanced Cyan Fluorescence Protein
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<222> LOCATION: (243)..(358)
<223> OTHER INFORMATION: Full-length VAMP2 with transmembrane domain and

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cleavage sites

<400> SEQUENCE: 6

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60

Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130 135 140

Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala Asp Lys Gln Lys Asn
145 150 155 160

Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
225 230 235 240

Gly Gly Met Ser Ala Thr Ala Ala Thr Val Pro Pro Ala Ala Pro Ala
245 250 255

Gly Glu Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg
260 265 270

Arg Leu Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met
275 280 285

Arg Val Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu
290 295 300

Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu
305 310 315 320

Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys
325 330 335

Met Met Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile
340 345 350

Ile Val Tyr Phe Ser Thr
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<212> TYPE: PRT
<213> ORGANISM: Rattus norvegicus

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1 5 10 15

Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu
20 25 30

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
35 40 45

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
50 55 60

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
65 70 75 80

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys Met Met
85 90 95

Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val
100 105 110

Tyr Phe Ser Thr
115

<210> SEQ ID NO 8
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<400> SEQUENCE: 8

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Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu
20 25 30

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
35 40 45

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
50 55 60

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser
65 70 75 80

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys Met Met
85 90 95

Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val
100 105 110

Tyr Phe Ser Thr
115

<210> SEQ ID NO 9
<211> LENGTH: 116
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

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1 5 10 15

Gly Gly Pro Pro Ala Pro Pro Pro Asn Leu Thr Ser Asn Arg Arg Leu
20 25 30

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val
35 40 45

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp
50 55 60

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser

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65                  70                  75                  80

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys Met Met
                85                  90                  95

Ile Ile Leu Gly Val Ile Cys Ala Ile Ile Leu Ile Ile Ile Ile Val
            100                 105                 110

Tyr Phe Ser Thr
        115
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What is claimed is:

1. A cell-based method of measuring protease activity of a Botulinum neurotoxin (BoNT) protease, comprising:

providing a transfected cell that produces a hybrid protein comprising a structure of A-B-C-D; wherein A is a transmembrane domain from synaptobrevin targeted to an intracellular vesicle membrane and is not cleavable by said BoNT protease, B is a first fluorescent protein, C is a BoNT protease recognition and cleavage sequence, and D is a second fluorescent protein selected to form a Forster resonance energy transfer (FRET) pair with said first fluorescent protein, and wherein a peptide spacer is disposed between one or more of C and B and C and D, wherein said peptide spacer and said BoNT protease recognition and cleavage sequence are selected to support FRET between said first fluorescent protein and said second fluorescent protein;

contacting said transfected cell with said BoNT protease under conditions to take up the BoNT protease by said transfected cell; and

measuring FRET between said first and second fluorescent proteins in said transfected cell.

2. The method of claim **1**, wherein said transfected cell is a cell selected from the group consisting of a neuronal cell, a neuroendocrine tumor cell, a hybrid cell, and a stem cell.

3. The method of claim **1**, wherein C comprises at least two of a BoNT/B, a BoNT/G, a BoNT/D, and a BoNT/F protease recognition and cleavage sequence.

4. The method of claim **1**, wherein C is a portion of synaptobrevin.

5. The method of claim **1**, wherein said peptide spacer comprises a length of equal to or less than 12 amino acids.

6. The method of claim **1**, further comprising a step of contacting said transfected cell with a putative BoNT inhibitor prior to contacting said transfected cell with said BoNT protease.

7. The method of claim **1**, wherein said hybrid protein having the structure of A-B-C-D comprises the amino acid sequence of SEQ ID NO: 2.

8. A cell-based method of measuring protease activity of a Botulinum neurotoxin (BoNT) protease, comprising:

providing a transfected cell that produces a first hybrid protein having a structure of A-C-B and a second hybrid protein having a structure of A-C-D; wherein A is a transmembrane domain of synaptobrevin targeted to an intracellular vesicle membrane and is not cleavable by said BoNT protease, B is a first fluorescent protein, C is a BoNT protease recognition and cleavage sequence, and D is a second fluorescent selected to form a FRET pair with said first fluorescent protein, wherein a peptide spacer is disposed between one or more of A and C, C and B, and C and D, wherein said transmembrane domain, said peptide spacer, and said BoNT protease recognition and cleavage sequence are selected to support FRET between said first fluorescent protein and said second fluorescent protein when said first hybrid protein and said second hybrid protein are collocated with a vesicle;

contacting said transfected cell with said BoNT protease under conditions to take up said BoNT protease by said transfected cell; and

measuring FRET between said first and second fluorescent proteins in said transfected cell.

9. The method of claim **8**, wherein said first hybrid protein comprises the amino acid sequence of SEQ ID NO: 4, and wherein said second hybrid protein comprises the amino acid sequence of SEQ ID NO: 6.

10. The method of claim **8**, wherein said transfected cell is a cell selected from the group consisting of a neuronal cell, a neuroendocrine tumor cell, a hybrid cell, and a stem cell.

11. The method of claim **8**, wherein C comprises at least two of a BoNT/B, a BoNT/G, a BoNT/D, and a BoNT/F protease recognition and cleavage sequence.

12. The method of claim **8**, wherein C is a portion of synaptobrevin.

13. The method of claim **8**, wherein said peptide spacer comprises a length of equal to or less than 12 amino acids.

14. The method of claim **8**, further comprising a step of contacting said transfected cell with a putative BoNT inhibitor prior to contacting said transfected cell with said BoNT protease.

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